substitution of glycine by valine in codon 116 of ACTH resulted in a profile of seizures, hypoglycaemia, impaired immune function and respiratory distress, characterized as "ACTH resistance syndrome."

4.9 eNOS, sulfate and red blood cells

Endothelial nitric oxide synthase (eNOS), which resembles cytochrome P450, plays a crucial rôle in providing the signaling molecule, nitric oxide, in the vasculature [150]. NO induces smooth muscle cell relaxation in the artery wall, leading to improved vascular flow. eNOS is dynamically regulated at the transcriptional, post-transcriptional and post-translational levels. Much has been written about eNOS's "pathological" production of superoxide under certain conditions, especially when the cofactor tetrahydrobiopterin (BH4) is depleted [151, 152]. Regulatory control of eNOS is complex, and, in particular, it only produces NO when it is both phosphorylated and detached from its secured scaffold to caveolin in lipid rafts in the plasma membrane [153]. Caveolin-1 prevents calmodulin binding under low calcium conditions [154, 155] Excess calmodulin, produced in response to calcium signaling, triggers the release of eNOS from its caveolin-bound site [156], and subsequent phosphorylation enables NO production.

In [157, 158], it was proposed that eNOS is a "moonlighting enzyme" which, when membrane-bound, rather than being inactive, produces sulfate, catalysed by sunlight. The superoxide is drawn into a zinc-occupied cavity created by the eNOS dimer, where it oxidizes sulfane sulfur, bound to conserved cysteine residues [159, 160] that encircle the cavity, to produce free sulfate. Details can be found in [157].

Red blood cells (RBCs) contain significant levels of eNOS, which is permanently located just within the plasma membrane. This has presented a puzzle to researchers, and some have even suggested that it is residual, because NO would be rendered ineffective through binding with haemoglobin, which would also disrupt oxygen transport [157, 161]. RBCs also steadily produce cholesterol sulfate, which plays an important rôle in maintaining their membrane negative charge and protects them from lysis and aggregation [162, 163]. Insufficient cholesterol sulfate leads to a high rate of haemolysis and shortened life span. Thus, RBCs plausibly use their eNOS to produce sulfate, which is then conjugated with cholesterol and exported to the external membrane wall.

eNOS is a member of a class of NOS isoforms that includes inducible NOS (iNOS) and neuronal NOS (nNOS). All known members of this class contain a conserved glycine residue (gly450), including all mammalian NOSs as wall as avian and insect NOS enzymes [164]. Gly450 is essential for NOS dimerization. Conservative amino acid substitutions at gly450 of murine iNOS abolishes NO production, dimer formation, and BH4 binding to the enzyme [165]. Furthermore, eNOS uniquely (compared to iNOS and nNOS) contains a myristoyl group covalently attached to the conserved N-terminal glycine, gly2, which is essential for securing eNOS to the membrane [164, 166]. It has been proposed that the myristoylating enzyme has an absolute specificity for glycine [23]. Experiments in which the glycine was replaced by alanine showed that neither myristoylation nor palmitoylation took place, and thus the defective enzyme only appeared in the cytoplasm [167–170].

It should be noted that other enzymes also have a conserved N-terminal glycine that supports myristoylation, including cyclic AMP-dependent protein kinase [171], calcineurin B [172], neurocalcin [173] and NADH–cytochrome b5 reductase [174]. Neurocalcin is found mainly in retinal photoreceptors and in neurons, where it is involved in the transduction of calcium signals [175]. Neurocalcin binds to clathrin, tubulin and actin in the cytoskeleton via myristoylation, and this suggests it may play a rôle in moderating clathrin-coated vesicle traffic [176]. This rôle would be disrupted if glyphosate replaces glycine at the N-terminus.

Thus, it becomes apparent that, if glyphosate is substituted for glycine at either the gly2 or the gly450 sites, eNOS will malfunction in both of its rôles of producing either sulfate or nitric oxide. This will have widespread pathological effects related to excessive haemolysis (anaemia), insufficient supply of cholesterol sulfate to the tissues, and insufficient production of NO, leading to vascular constriction and hypertension. Disruption of iNOS function will lead to impaired immunity, since iNOS defends the host against infectious agents [177]. And, of course, other important enzymes that also support myristoylation via a terminal glycine will behave in unpredictable ways when that glycine is replaced with glyphosate.

4.10 Arylsulfatases

Arylsulfatases are a family of enzymes that remove sulfate from sulfated molecules. Substrates include: the sulfated glycosaminoglycans—keratin sulfate, chondroitin sulfate and heparan sulfate; the sulfated sterols—cholesterol sulfate, estrone sulfate, testosterone sulfate, DHEA sulfate etc.; sulfated phenolic compounds; and the sulfated lipids such as sulfatide (sulfated galactocebroside). A defective version of arylsulfatase A, which removes sulfate-21 from sulfatide, results in the

condition of metachromatic leukodystrophy [178]. The infantile form of this genetic disease is characterized by muscle wasting and weakness, muscle rigidity, developmental delays, blindness, convulsions, impaired swallowing, paralysis and dementia. Life expectancy is below five years.

All members of the arylsulfatase family are subject to a unique modification that is necessary for activation, involving the transformation of a cysteine residue into formylglycine (FGly) [179]. In a rare inherited disorder named multiple sulfatase deficiency (MSD), the activities of all sulfatases are severely reduced. This disorder involves an impairment in the transformation of cysteine to FGly. A highly conserved motif consisting of four amino acids (LTGR) is found in all human and microbial arylsulfatases, near the modified cysteine. A 16-mer segment including this motif is essential and sufficient for the formation of FGly [180]. It is likely that the conserved glycine residue in the motif is essential to support the flexibility needed to present the cysteine to the modifying enzyme [181]. Without this transformation, the enzyme is completely inactive. Therefore, displacement of this glycine by glyphosate would likely disrupt enzyme activation.

In a mouse model of autism, maternal immune activation through polyinosinic:polycytidylic acid (poly(I:C)) injection produced offspring with characteristic features of mouse autism [182]. Likely due in part to a leaky gut, these offspring had sharply elevated serum levels of 4-ethylphenylsulfate, produced by the gut microbes, with a 46-fold increase over controls. Injection of 4-ethylphenylsulfate into normal mice induced autistic behaviour. It is plausible that impaired phenol sulfatase activity, particularly in the context of a leaky gut, would cause the accumulation of sulfated phenols in the plasma, contributing to autism.

5. NEURODEGENERATIVE DISEASES

We have already seen that the pathology of Alzheimer's disease is linked to overexpression of GSK3, which can be induced by the substitution of negatively charged amino acids in place of glycine in the N-terminal region. Glyphosate is negatively charged at biological pH.

Beyond Alzheimer's, multiple neurodegenerative diseases are associated with aggregated and tangled proteins including Lewy bodies, tauopathies, senile plaques and neurofibrillary tangles. In this section, we will focus on four classes of neurodegeneration that can be linked to disruption of conserved glycines in specific misfolded proteins: prion diseases, Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (ALS). In all four of these cases, it has been determined that rare soluble non-fibrillar forms of the aggregated proteins are much more damaging than the insoluble precipitates. It has also been shown that conserved glycines support the flexibility that is needed to allow the hydrophobic components of the molecule to assemble so as to precipitate out of aqueous solution. Glycine is hydrophobic, whereas glyphosate is amphiphilic, and it is also much bulkier than glycine. Glyphosate's solubility would likely be higher in the cytoplasm of a cell than in serum both because of the higher pH and because of cationic buffering by potassium. In fact, potassium salts are used in glyphosate formulations to increase its solubility. It seems plausible that the rare soluble non-fibrillar forms of aggregated proteins that are toxic have glyphosate in place of glycine in their structure.

5.1 Prion diseases

Prion diseases, also called transmissible spongiform encephalopathies, are novel degenerative diseases in which the infective agent is a misfolded protein. Prions are believed to be responsible for Kuru, Creutzfeldt-Jakob disease, and bovine spongiform encephalopathy (BSE, mad cow disease). BSE first appeared in the United Kingdom in 1986, after glyphosate had been used to control weeds in animal feed for at least a decade. While BSE is believed to be caused by feed contaminated with the brain, spinal cord or digestive tract of infected carcasses, there remains the open question of what caused the original appearance of misfolded proteins to initiate the infection. Prion proteins contain a glycine-rich hydrophobic region that shows almost perfect conservation across a wide range of species. This region appears to be important for the misfolding process and prion propagation [183]. It seems remarkable that a highly conserved region of the protein, unaltered by genetic mutations, could be the source of the toxicity. The normal form of prion proteins, PrP^{C}, is rapidly catabolized, whereas a pathogenic isoform, PrP^{Sc}, is highly resistant to proteolysis [184]. A subsequence containing only PrP 106–126 is a highly conserved unstructured region of PrP, which is considered to be the main contributor to fibrillogenicity. It has a high tendency to aggregate into a β-sheet structure forming amyloid fibrils *in vitro* [185, 186].

There is controversy regarding whether the toxicity is due mainly to mature fibrils or to protofibrillar aggregates. A definitive study [187] showed that two strictly conserved glycine residues, at positions 114 and 119, within the highly conserved region, are the main drivers behind fibril formation, likely due to the high flexibility that they introduce in the molecular structure. If either of these is substituted by glyphosate, fibril formation would be impaired, due to the decreased flexibility. Remarkably, although replacement of these

glycines with alanine interfered with aggregate formation, it produced a higher concentration of a soluble non-fibrillar form which was, however, extremely neurotoxic [184]. Alanine has an additional methyl group, which makes it a bulkier molecule than glycine, restricting flexibility of the assembled protein. Glyphosate substitution for glycine would be expected to be even more disruptive than alanine, given its additional methylphosphonyl group. The lack of flexibility to organize the hydrophobic unit into a fibril will favour the toxic soluble form of the peptide. Furthermore, glyphosate can be expected to resist enzymatic degradation, and glyphosate-containing peptoids would also be resistant to proteolysis, in both cases due mainly to the highly stable C–P bond [188].

5.2 Alzheimer's disease, prions and β-amyloid

Alzheimer's disease is the most common form of dementia, accounting for 60% to 80% of all cases [189]. Worldwide, the prevalence of dementia was more than 35 million in 2010, and projected to be more than 65 million by 2030 and 115 million by 2050 [190]. The incidence of Alzheimer's disease is increasing at an alarming rate in the United States, in step with the dramatic rise in the use of glyphosate on corn, soy and wheat crops [30]. β-amyloid (Aβ) is now well established as a causal factor in Alzheimer's disease, although the mechanism of toxicity remains controversial [191]. The Aβ that accumulates in the Alzheimer's brain consists of deposited insoluble fibrillar components, monomers, and soluble oligomers, the latter being the most toxic form. The levels of the monomer and the deposited precipitates are orders of magnitude greater than the levels of the toxic soluble oligomers, which are known to cause both acute synaptotoxicity and neurodegeneration [190]. The pharmaceutical industry has developed immunotherapies that target Aβ, but none of them are specific to the toxic soluble form, and this likely explains their lack of efficacy [192]. The challenge to the industry is to develop a drug that uniquely targets the soluble oligomers.

Growing evidence supports the concept that soluble non-fibrillar forms of Aβ are the most toxic, and their toxicity can be mimicked by a synthetic peptide containing the first 42 residues (Aβ42) [193]. Interestingly, Aβ has a GXXXG domain with conserved glycines at positions G29 and G33 [194]. Substitution of alanine in place of glycine at residues G29 and/or G33 led to an attenuation of dimerization, and specifically increased the formation of Aβ38 and shorter species at the expense of Aβ42. Munter et al. argued that the glycines promote dimerization and that this impedes access of proteases to the molecule, resulting in the survival of the longer peptide chain. However, it is extremely unlikely that a highly conserved element in the protein could be responsible for disease. An alternative thought is that glyphosate substitutes for glycine, increasing solubility and preventing proteolysis. This is in line with work that has shown that aminopeptidases can be disrupted by methylphosphonic acid [10]. It can be envisioned that the presence of glyphosate in place of glycine upstream interferes with the stripping off of residues 41 and 42 by γ-secretase, leaving behind a soluble and damaging Aβ42 peptide.

Magnesium deficiency has been linked to Alzheimer's disease [195, 196], and *in vitro* studies have shown that low magnesium leads to increased production of Aβ [197]. Glyphosate's chelation of +2 cations can be expected to deplete magnesium availability, and studies on soy have shown that glyphosate interferes with magnesium uptake in plants [198, 199]. The effect of low magnesium will work synergistically with glyphosate's inclusion in the Aβ peptide to induce Alzheimer's disease (AD).

Bush, Cherny and others note that zinc, copper and iron accumulate in brain plaques [200–204]. Aβ is a Zn and Cu metalloprotein, and zinc has been shown to induce amyloid formation in Aβ [200]. Glyphosate strongly chelates Cu, as well as Zn, and ferrous iron, Fe^{2+}, which, as Monsanto's John E. Franz notes, quickly oxidizes to the ferric form, Fe^{3+}. Metal chelate formation constants show strong binding potential for these elements at 11.9, 18.2 and 6.9 for Cu, Zn and Fe respectively, as compared to the parent amino acid glycine at 8.6, 5.4 and 4.3 respectively. Maynard et al. (2005) assert: "Aβ and APP (amyloid precursor protein) expression have both been shown to decrease brain copper (Cu) levels, whereas increasing brain Cu availability results in decreased levels of Aβ and amyloid plaque formation in transgenic mice. ... Interestingly, the highest levels of free or synaptic Zn are found in cortex and hippocampus, the regions most affected in AD. Zn^{2+} reuptake after synaptic release is a rapid, energy-dependent process. Hence, energy depletion could cause a pooling of extracellular Zn^{2+}, contributing to Aβ deposition" [203]. Glyphosate's disruption of COX could impair energy supplies, leading to excess Zn^{2+} accumulation. Religa et al. show that zinc levels rise with tissue amyloid levels and "were significantly elevated in the most severely demented cases (CDR 4 to 5) and in cases that had an amyloid burden greater than 8 plaques/mm^2. Levels of other metals did not differ between groups." They concluded that the zinc accumulation is dominant in cases of advanced Alzheimer's disease and linked to brain amyloid peptide accumulation as well as to the severity of the disease [204]. Such a pairing of these elements with the amino acid glyphosate in amyloid protein would likely

form misfolded proteins as well as insoluble plaques due to the known resistance of the analogue to proteolysis.

Because of its small ionic radius and strong positive charges, aluminum firmly binds to metal-binding and phosphorylated amino acids, acting as a cross-linker by binding multiple amino acids simultaneously [205], which can cause the oligomerization of proteins, inhibiting their degradation by proteases. This is believed to be a mechanism for the neurofibrillary pathology of phosphorylated tau protein [206, 207]. We have already established that glyphosate likely induces excess protein phosphorylation due to its excitatory effects on kinases and inhibitory effect on phosphatases. However, glyphosate itself also binds aluminum, particularly through oligomeric complexation of an aluminum ion [208]. Thus, two molecules of a peptoid/peptide, both of which contain glyphosate, will likely become linked together via an aluminum ligand binding two embedded glyphosate residues, one in each peptide. This would almost surely lead to impaired protein degradation and accumulation of fibrils. *In vitro* studies have shown that soluble dimeric and oligomeric forms of Aβ are more toxic than monomeric Aβ [209, 210].

Increasingly, prions are suspected of playing a rôle in Alzheimer's disease. A recent, well-designed study has demonstrated that a triad formed from amyloid-β, PrP^C and a metabolic glutamate receptor is critical for the disruption of synaptic plasticity by the soluble non-fibrillar forms of Aβ [211]. High affinity binding of Aβ to PrP has been localized to the region of PrP from residue 91 to residue 119 [212]; within this region, residues 114 and 119 are the two conserved glycines in PrP [184].

5.3 Cataracts and Alzheimer's disease

Crystallin is the dominant protein found in the lens of the eye. Cataract formation is the result of amyloid protein aggregation from crystallins, which results in insoluble β-amyloid deposits in the lens [213]. Post-mortem studies on Alzheimer's patients revealed that Aβ is also present in the cytosol of cells from the lenses of people with Alzheimer's disease and that it is associated with cataracts [214]. In fact, amyloid plaques in cataracts and in the brain in Alzheimer's patients were identical. Furthermore, α-B-crystallin is found in association with brain plaques and fibrillary tangles in Alzheimer's, Creutzfeldt-Jakob and Parkinson's diseases.

An increase in phosphorylation of crystallin is linked to increased cataract risk [215]. Such an increase can be expected in the context of hyperactive kinases and inhibited phosphatases, such as is expected with glyphosate insertion in place of glycine in these molecules. Furthermore, a single mutation of the conserved glycine-98 residue of crystallin to arginine results in a defective form of the protein that lacks chaperone function, and is susceptible to heat-induced aggregation [216]. This mutation is also linked to increased risk of cataracts. The α-crystallins in particular play an important rôle in chaperoning crystallins to prevent protein aggregation and precipitation. Thus, it appears that alterations to glycine residues can play a rôle in cataracts that is completely analogous to the rôle they play in Alzheimer's disease, and the two conditions are closely linked.

Perhaps unsurprisingly, given these cataract risk factors linked to defective crystallin, Monsanto's own early rodent studies found a link between glyphosate exposure and cataract formation [29]. Monsanto's 1990 (Stout & Ruecker) chronic rat exposure study found significant incidence of y-sutures and other ophthalmic degenerative lens changes caused by glyphosate. The pathologist for the study, Dr Lionel Rubin, noted in his ophthalmoscopic examination report that: "There appears to be a dose-related occurrence of cataract affecting male group M3. The type of cataract affecting this group is the diffuse posterior sub-capsular type and to a lesser extent, anterior polar and sutural types." Displacement of pupils and ocular opacities in the presence of glyphosate was also noted in 1983 by Knezevich and Hogan [29].

5.4 α-Synuclein and Parkinson's disease

A 35-amino-acid peptide was isolated from the insoluble core of Alzheimer's disease amyloid plaque, and was found to be a fragment of α-synuclein, a neuronal protein of unknown function. This fragment had a striking sequence similarity with the carboxyl terminal of Aβ, as well as a region of PrP implicated in amyloid formation [217]. α-synuclein aggregates are found in association with Lewy bodies present in Parkinson's disease patients, and is also linked to dementia and multiple system atrophy [218, 219]. A novel ELISA test has been developed that detects only oligomeric soluble aggregates of α-synuclein in the blood. It was shown that 52% of Parkinson's disease patients tested positive as against only 15% of controls [220]. A 9-residue sequence, ^{66}VGGAVVTGV^{74}, containing three glycine residues, has been shown to be crucial for the fibrillization and cytotoxicity of α-synuclein [221]. Fibrillization and cell toxicity are completely eliminated when this sequence is deleted.

5.5 TDP-43 and ALS

Transactive response DNA binding protein 43 (TDP-43) is a transcriptional repressor that binds both DNA and RNA, and has multiple other functions, including pre-

mRNA splicing and translational regulation. Exon 6 of TDP-43 encodes a C-terminal glycine-rich domain where multiple missense mutations have been implicated in association with amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD), a subtype of dementia [222]. TDP-43 is now considered to be the signature class of inclusional lesions for sporadic ALS. TDP-43 is also recognized for its ability to repress HIV transcription [223].

The C-terminus of TDP-43 bears sequence similarity to prion proteins. Synthetic peptides near residue 315 form amyloid fibrils *in vitro* and cause cultured neuronal death [224]. Accumulation of protease-resistant fragments may spread the disease phenotype among neighbouring neurons, similar to the pathology associated with prion diseases.

TDP-43 is a member of a class of ribonucleoproteins known as 2XRBD-Gly proteins. The class share the common feature of a glycine-rich C-terminus that probably serves a similar function in all the members of the class. Among 53 unrelated sporadic or familiar ALS cases, two of whom suffered from concurrent FTLD, 29 different missense mutations in TDP-43 have been reported [222]. All but one of them occurred in the C-terminal glycine-rich domain of exon 6. The subset of these mutations that involve a substitution for glycine are concentrated in the region between residue 275 and 310, the most glycine-dense region of the C-terminus. Thus, replacing glycine with any other amino acid increases risk to ALS. Non-genetic replacement with glyphosate can be expected to have a similar outcome.

About 20% of patients with familial ALS have mutations in Cu,Zn superoxide dismutase (SOD). One of the more common mutations found is a substitution of alanine in place of glycine at gly93, which introduces a modest gain of function [225]. Although this change appears to have little effect on enzyme activity, transgenic mice with this genetic mutation become paralysed in one or more limbs as a result of motor neuron loss in the spinal cord and do not live beyond five or six months. Clearly, substitution of a bulkier molecule in place of glycine disrupts the function of the enzyme in ways that are not yet understood.

6. MICROBIOME DISRUPTION AND IMMUNE SYSTEM IMPAIRMENT

In this section we discuss several examples of proteins that play a rôle either in maintaining the health of the gut microbiome or in human defence against microbial infection. In each case, conserved glycines are essential for protein function. We begin with a section on the disruption by glyphosate of PEP carboxylase, which has major impact on microbial health, as this enzyme is central to both carbon fixation and nitrogen fixation. The next section describes glycine riboswitches and their rôle in the metabolization of glycine in the medium via the glycine cleavage system. This is important both to detoxify glycine and to supply methyl groups for one-carbon metabolism. Antimicrobial peptides such as α-defensin are important for human immune function, and these proteins contain conserved glycines. Finally, HIV-AIDS infection is linked to impaired phosphatase activity, particularly a constitutively expressed tyrosine phosphatase that is highly expressed in T-cells.

6.1 Nitrogen fixation and PEP carboxylase

Mung beans exposed to glyphosate at levels appropriate for weed control show reduced fixation of nitrogen into organic matter [226]. Nitrogenase, an essential enzyme in plants for nitrogen fixation, converts nitrogen gas to ammonia, which is then conjugated with glutamate to produce glutamine. A study on lupins showed that glyphosate exposure, even at sublethal levels, severely inhibited nitrogenase activity, resulting in a decrease in starch content and an increase in sucrose content. The practice of using glyphosate as a pre-harvest ripener in sugar cane to increase yield exploits this property of increased sucrose production [227]. The mechanism was traced to inhibition of phosphoenol pyruvate carboxylase (PEPC), subsequent to accumulation of shikimate via blockage of the shikimate pathway [228]. PEPC plays an essential rôle in the incorporation of both CO_2 and nitrogen into plants [229, 230].

PEPC's regulation is controlled by levels of shikimate rather than through product inhibition. Since PEP is the input to both PEPC and 5-enolpyruvylshikimic-3-phosphate synthase (EPSPS), the step in the shikimate pathway that glyphosate disrupts, PEP accumulates at ever greater levels while both the carboxylase pathway and the shikimate pathway are blocked. Most of the carbohydrate pool is then exhausted through conversion to shikimate, acting as a metabolic sink. Shikimate accumulates to very high levels due to glyphosate's inhibition of EPSPS, while the synthesis of aromatic amino acids, normally derived from shikimate, is blocked.

At the extreme C-terminus of PEPC there is an invariant glycine residue which plays an essential rôle in enzyme activity [231]. Even the conservative replacement with alanine (one extra methyl group) leads to loss of function both *in vivo* and *in vitro*, with an experimentally demonstrated drop to only 23% of the wild type activity level in sorghum [231]. In experiments on *E. coli*, perturbation of the terminal gly-961 by either

conservative neutral substitution with alanine or valine or even by specific deletion did not seem to cause any apparent harmful effects. However, replacement with a negatively charged amino acid such as aspartate resulted in a complete shutdown of enzyme activity. The authors wrote: "PEPC appears to not tolerate additional negative charge at its extreme C-terminus beyond that of the main chain free CO_2^- group."

Glyphosate substitution would of course represent the introduction of additional negative charge. Thus, it seems almost certain that glyphosate substitution for glycine at this conserved terminal site would severely inhibit the enzyme's activity, beyond any inhibition already induced by the build-up of shikimate. This offers a further explanation for the empirically observed suppression of PEPC by glyphosate, and it also suggests that glyphosate disrupts nitrogen fixation [232].

6.2 Glycine riboswitches

Glycine is both essential and toxic to bacteria. It is well known that glycine inhibits bacterial growth [233–236], by substituting for alanine into peptidoglycan precursors [237–239]. Glycine-containing precursors are poor substrates for peptidoglycan biosynthesis enzymes as well as for the transpeptidation reaction, leading to both a deficiency in muropeptides and a high percentage of muropeptides that are not cross-linked. These modifications to the cell wall severely restrict growth.

As a consequence of glycine's toxicity, it is important for bacteria to be able to quickly break glycine down into basic building blocks. Oxidative cleavage to CO_2, NH_4^+ and a methyl group is carried out by the glycine cleavage system (GCS), and the methyl group becomes a major source for one-carbon metabolism, beginning with the conversion of tetrahydrofolate (THF) to methylene-THF [237], which is then used to biosynthesize various cellular compounds, including, importantly, purines and methionine. The GCS also produces NADH in the oxidative cleavage step, which yields energy through the electron transport system. As well the GCS is the most prominent pathway for serine and glycine catabolism in humans [240]. Mutations in GCS-encoding genes are linked to defects in neural tube development, causing spina bifida and anencephaly [241, 242, 243].

Riboswitches are small non-coding RNA segments typically located in the 5' untranslated regions (UTRs) of bacterial mRNAs, and they serve as both sensors of cellular metabolites and effectors of regulatory responses. Studies have revealed the presence of glycine riboswitches in the 5' UTRs of the enzymes involved in the GCS [244]. These riboswitches bind directly to glycine and turn on the genes for transcription of enzymes needed to metabolize it. In this way, glycine is quickly cleared and put to good use, fueling the electron transport chain and the one-carbon metabolism pathways. Glycine is highly toxic to mutants missing these riboswitch regions; a medium containing only 1% glycine severely restricts their growth [237].

Glyphosate is a patented antimicrobial agent, and its toxicity to humans has been attributed in part to its adverse effect on the microbiome [26]. In addition to other actions such as metal chelation and inhibition of the shikimate pathway, glyphosate, acting as a glycine analogue, disrupts the glycine regulatory system and cell wall construction. Glyphosate perhaps, like glycine, substitutes for alanine in the peptidoglycans. Glyphosate likely also binds to the glycine riboswitches, acting as a glycine analogue, and it could interfere with the signaling mechanism due to its altered structure and negative charge.

6.3 α-Defensin and antimicrobial peptides

Human α-defensins are important members of a broad class of antimicrobial peptides that are found throughout the tree of life [245, 246]. All of the human α-defensins, although their molecular structures are quite variable, contain a conserved glycine, gly17, which is part of a β-bulge structure. Gly17 is in fact the only non-cysteine residue that is invariant in α-defensins. Gly17 is part of a larger structural motif known as the γ-core, which is present across many classes of antimicrobial peptides. When other amino acids are substituted for gly17, dimerization is impaired, and this disrupts the ability to self-associate, inhibit anthrax lethal factor, and kill bacteria [247].

Even the conservative substitution of L-alanine for glycine inhibits protein function. Bulkier hydrophobic side chains are likely to create steric clashes, a polar side chain might introduce hydrogen bonds, and a charged side chain might invite electrostatic attraction or repulsion [247]. Thus the methylphosphonyl group in glyphosate in place of the conserved glycine is likely to have a major negative impact on the protein's effectiveness against microbes.

6.4 HIV-AIDS

Protein tyrosine kinases (PTKs), acting in concert with protein tyrosine phosphatases (PTPases), control levels of cellular protein tyrosine phosphorylation. Changes in tyrosine kinase and phosphatase activity are implicated in numerous human diseases, including cancer, diabetes and pathogen infectivity [248].

Impaired phosphatase activity due to disruption of a conserved glycine may play a rôle in increasing HIV infectivity. c-Jun N-terminal kinases (JNKs) are signaling

kinases that respond to mitogen-activated protein (MAP) kinase signaling and regulate many cellular activities. JNKs are activated through dual phosphorylation of threonine and tyrosine residues, and inactivated by matched phosphatases [249]. JNK activation is implicated in HIV infections. Quiescent (resting) human peripheral blood T lymphocytes do not support efficient HIV infection, both because reverse transcription takes longer and because of impaired integration of the viral complementary DNA [250]. Cellular JNK is only expressed following activation, and it regulates permissiveness to HIV-1 infection. In JNK-activated T lymphocytes, viral integrase is phosphorylated by JNK on a highly conserved serine residue in its core domain. This modification is required for efficient HIV-1 integration and infection. As a consequence, it is mainly the activated lymphocytes that are infected.

A dual-specificity PTK that can also dephosphorylate threonine/serine residues is human tyrosine phosphatase vaccinia H1-related (VHR). This phosphatase has special significance because it is highly expressed in T-cells, and it is expressed constitutively rather than in response to a signaling cascade [130]. VHR has a conserved glycine residue within the protein tyrosine phosphatase (PTP) loop, which maintains its flexibility and is essential for substrate binding and enzymatic activity [251]. Substitution of either proline or alanine for the conserved G127 residue resulted in mutants with a decrease in catalytic activity of about 400-fold, and the K_i value was increased by 38-fold with alanine and 19-fold with proline [130].

VHR may play a significant rôle in protection from HIV infection due to its constitutive expression in T-cells [252]. VHR is a negative regulator of the Erk and JNK pathways in T-cells. Only constitutively expressed enzymes are present in the early phase immediately following MAP kinase activation. VHR is the only known MAP kinase-specific phosphatase that is constitutively expressed in lymphocytes. It can thus immediately dephosphorylate activated JNK and in this way protect from HIV infection.

It is likely that glyphosate's disruption of VHR and other protein phosphatases with conserved glycines has implications far beyond HIV infection, since protein phosphorylation status plays such an important rôle in signaling cascades. In fact, the combination of activation of kinases and suppression of phosphatases that can plausibly be induced through glyphosate's displacement of conserved glycines in the enzymes can be predicted to lead to an overabundance of phosphorylated molecules, systemically. This may contribute to the recent antiphospholipid syndrome epidemic. It may also play a rôle in cancer: tyrosine kinase inhibitors are often used to treat cancers with aberrant tyrosine kinase receptor activity [253].

7. EFFECTS ON SPECIFIC ORGANS

In this section we examine proteins with conserved glycines, where substitution with glyphosate can explain porphyrias and liver disease, renal failure due to impaired iron uptake (leading to simultaneous iron toxicity and iron deficiency), disruption of cytochrome P450 enzymes and glaucoma, impaired collagen function leading to osteoporosis and increased risk to bone fracture, and malignancy in non-Hodgkin's lymphoma due to defective binding of tumour cells to dendritic cells.

7.1 Porphyrias and liver disease

Gly232 is a strictly conserved residue in the enzyme protoporphyrinogen oxidase (PPOX). A paper from 1997 discussed three patients with a missense point mutation substituting arginine in place of this glycine residue. This led to a deficiency in PPOX activity, resulting in impaired haem synthesis and variegate porphyria [254].

In a mouse model of porphyria, it was shown that mice developed fatty liver disease due to the accumulation of protoporphyrin in the liver and resulting induction of oxidative stress. The model involved excessive inhibition of KEAP1-mediated Nrf2 degradation, resulting in upregulation of the expression of keratin and the appearance of keratin-rich Mallory–Denk bodies [111].

It seems possible that, in humans, glyphosate substitution for glycine in PPOX would lead to a non-genetic expression of porphyria, and glyphosate substitution for glycine in KEAP1 would interfere with KEAP1's ability to suppress the overexpression of Nrf2. This model would explain protoporphyrin-induced fatty liver disease in the context of glyphosate exposure, progressing to cholelithiasis, end-stage liver disease and liver failure [255].

7.2 Siderophores and renal failure

Siderophores are small iron-chelating compounds secreted by microörganisms as a mechanism to solubilize insoluble ferric iron compounds [256]. The class of enzymes that imports these siderophores is important both for iron uptake and for uptake of vitamin B_{12}. These enzymes contain two conserved glycines, and these are the only invariant residues found in every enzyme in this family of iron transport proteins [257]. Substitution of alanine for glycine was better tolerated than substitution of larger amino acids. This suggests that glyphosate substitution would induce impaired iron uptake as well as impaired vitamin B_{12} uptake in *E. coli* and other microbes.

In *Bacillus subtilis*, an important microbe in the human micriobiome, iron deprivation induces upregulation of all the enzymes involved in the synthesis of the iron siderophore, bacillibactin [258], including the enzymes needed to synthesize glycine, a precursor to bacillibactin. Glyphosate has been shown to inhibit growth in tumour cells, and the proposed mechanism was inhibition of glycine synthesis from serine, through its action as a glycine analogue [17]. Thus, it can be expected that both siderophore synthesis and iron-loaded siderophore uptake will be impaired in the presence of glyphosate. Glyphosate also chelates iron, making it unavailable.

In our previous work [29] we discussed details of a Monsanto study by Lankas and Hogan (1981), which found microscopic changes of the kidney associated with chronic progressive nephropathy. Focal tubular hyperplasia and focal tubular dilation, which precede acute tubular necrosis and nephrosis, were detailed. Acute tubular necrosis (ATN) is a life-threatening syndrome caused by impaired function of the proximal tubule of the kidney [259–261]. This is the form of kidney failure that characterizes the alarming epidemic of kidney disease among agricultural workers in Sri Lanka and elsewhere, which has been linked to glyphosate working synergistically with toxic metals [262]. It has been found through experiments in mice that defective iron uptake from siderophores in the proximal renal tubule can cause simultaneous iron deficiency and iron toxicity, explaining the disease process [263]. Unbound iron forms reactive ferryl or perferryl species [264] which can damage lipids, nucleotides and the DNA backbone [265, 266]. Remarkably, Mori et al. [263] showed that the proximal tubules markedly upregulate synthesis of lipocalin, a protein that specifically functions to take up microbial siderophores bound to iron, under stress conditions. In fact, the tubules appear to rely on microbial siderophores to supply their iron. A GXW motif is conserved in all members of the lipocalin family [267]. Hence, it can be expected that impaired siderophore synthesis by microbes, combined with impaired uptake in the renal tubules due to glyphosate substituting for conserved glycines in lipocalin, can lead to destructive oxidative damage by free iron paradoxically combined with iron deficiency. Because several enzymes involved in amino acid biosynthesis are iron-dependent, iron deficiency causes amino acid starvation [258], further stressing the renal tubules.

Transferrin-based iron uptake is likely to also be disrupted by glyphosate, and this can help explain the worldwide iron deficiency anemia epidemic, linked to both impaired brain development [268] and obesity [269]. A recent study investigated the rôle of the conserved sequence of four glycines in the protein responsible for uptake of iron from *human transferrin* in the infective agent, *Neisseria gonorrhoeae* [270]. The four glycines follow a hydrophobic lipid anchor region that secures the molecule in the membrane. While deletion of the glycines did not prevent anchoring in the membrane, it did interfere with the uptake of iron from transferrin, suggesting impairment of the flexibility needed to form the iron chamber, which allows for efficient iron internalization through the β-barrel. It can be anticipated that this protein and others similarly designed in other species, concerned with mineral uptake, would be impaired by glyphosate substitution for conserved glycines.[1]

7.3 Cytochrome P450 enzymes and glaucoma

Studies on rats have shown that glyphosate suppresses the activity of cytochrome P450 enzymes (CYPs) in the liver [26, 273]. In a hinge region of CYP1B1, characteristic of microsomal CYPs, a proline- and glycine-rich region follows the N-terminal transmembrane domain. It has been proposed that the proline-proline-glycine-proline motif joins the membrane-binding N-terminus to the globular region of the P450 protein [274]. The hinge permits flexibility between the membrane-spanning domain and the cytoplasmic portion of the molecule [275]. Mutations in the hinge regions interfere with the proper folding and haem-binding of CYPs [275, 276].

Mutations in CYP1B1 have been closely linked to primary congenital glaucoma [277, 278]. In a study involving 24 Saudi Arabian families, the most common mutation was a G → A transformation at nucleotide 3987, occurring in 78% of the chromosomes analysed [277]. This results in substitution of glutamate for gly61 in the hinge region. Gly61 is one of the most highly conserved residues in this region.

Another study involving five families with primary congenital glaucoma in Saudi Arabia identified 2 out of 8 missense mutations that involved glycine being replaced by another amino acid, one being the gly61 glu mutation [278]. The second mutation involved a substitution of tryptophan for glycine in helix J in the 5' end of exon 3, part of the core structure of the enzyme. Both mutations were associated exclusively with the glaucoma phenotypic expression. It is possible that glyphosate substitution for glycine at these two conserved residues contributes to

[1] A pattern of glycine-rich regions near hydrophobic sequences occurs repeatedly in protein design, and is probably necessary for flexibility near the membrane anchor region [271, 272].

glyphosate's observed suppression of CYP enzyme activity, more generally.

7.4 Collagen, bone fractures and osteoporosis

Glycine is the most common amino acid in collagen, making up one third of the total amino acid residues in the molecule. Over 10% of the molecule consists of a helical region, where each coil in the triple helix is made up of glycine-led triplets of the form $(gly\text{-}2\text{-}3)_n$ [279]. Proline and hydroxyproline are also highly overrepresented in collagen, and they appear in over half of the glycine-led triplets. Triple helix formation is essential for the transport of type I procollagen out of the ER for secretion to form extracellular matrix fibrils to support mineral deposition in bone [280].

Osteogenesis imperfecta (OI), which is also known as brittle bone disease, is a congenital bone disorder characterized by a strong predisposition towards bone fractures. The condition is caused by genetic mutations in collagen, mainly collagen I. Overwhelmingly, these mutations concern substitutions for glycine in the glycine triplet regions [281–283]. One third of the glycine mutations that occur in the alpha chain of collagen 1 are lethal, especially when the substituting amino acid is electrostatically charged or has a side branch [284]. The lethal regions align with proteoglycan binding sites, suggesting impaired proteoglycan attachment. The majority of the substitutions involve glycine residues in the triple helical domain. Mutations have been found that account for all of the possible amino acid substitutions for glycine, except the stop codon, that can be produced by changing just one nucleotide in the DNA code for glycine.

A case of a male child in which glycine was replaced by tryptophan (the only case known for this substitution) in a residue on the α2 chain demonstrated a severe phenotype characterized by numerous fractures already present at birth, and numerous additional fractures occurring postnatally. By the age of 9 years his height was below the 3rd percentile, he suffered from generalized osteoporosis, and had a large skull, thin ribs, a severely deformed pelvis, and markedly deformed long bones [283]. It is thus likely that random replacements of any of the multiple glycine molecules in collagen with glyphosate would also disrupt collagen's structure, leading to osteoporosis as well as a sensitivity to bone fractures, which might in part explain "shaken baby syndrome" [285]. Osteoporosis is also a modern epidemic [286]: As of 2003, osteoporosis affected one in three women and one in twelve men worldwide [287]. We are witnessing an increase in age-specific fracture rates due to an unknown aetiology.

7.5 Non-Hodgkin's lymphoma

Non-Hodgkin's lymphoma (NHL) has been linked to glyphosate in occupational exposure studies [288, 289]. The tumour cells of NHL patients appear to be neoplastic versions of activated B cells, in that they both express very late antigen-4 (VLA-4), which binds to vascular cell adhesion molecule-I (VCAM-1) expressed on follicular dendritic cells, and in this way traps the dendritic cells. This binding mechanism is central to the generation of the immune response, and it influences activation and proliferation of immune cells. Blocking studies demonstrated that the binding of follicular lymphoma cells to malignant follicles was inhibited with anti-VLA-4 and anti-VCAM-1 antibodies [290]. The VLA-4 from malignant cells studied from different patient populations demonstrated variable and weakened ability to bind to VCAM-1, and it was proposed that defective binding might be the factor that induces malignancy. The authors suggested that lower adhesive capacity might explain the tendency of neoplastic cells to disperse: "Therefore, a deregulated or dysfunctional VLA-4:VCAM-1 interaction in follicular NHL may be similarly important to the proliferation of the neoplastic cells" [290].

VLA-4 is required for normal development of both T- and B-cells in the bone marrow, in part by regulating the balance between proliferation and differentiation of haematopoietic progenitors [291]. It can therefore be expected that impaired function would lead to pathologies such as immune dysfunction and cancer. Two conserved glycine residues at positions 130 and 190 are essential for its adhesive activity [292]. Glyphosate's link to NHL may therefore be explained through substitution of glyphosate for glycine at one or both of these conserved residues.

8. NEURAL TUBE DEFECTS AND AUTISM

Glyphosate can penetrate past the placenta [293]. Alarming increases in birth defects such as microcephaly, anencephaly, cleft palates and other facial defects have been found in regions of South America and Paraguay where glyphosate is used extensively on core crops [294, 295]. The US Centers for Disease Control have reported on an excessive number of anencephaly births in Yakima (Washington), at four times the national average rate [296]. This increase coincided with a large increase in the use of glyphosate to control waterway weeds.

A recent study by Roy et al. on zebrafish embryos revealed that glyphosate causes microcephaly in zebrafish, and that the forebrain and midbrain are affected (but the hindbrain was spared) [297]. A US-based study found that the cerebellum is frequently disproportionately large in human microcephaly,

particularly in the more severe cases, reflecting a larger effect on the forebrain compared to the hindbrain [298].

A study on tadpoles conducted by Carrasco et al. involved dilutions of 1/500,000 of glyphosate formulations. [299]. They showed several pathologies in development that relate to neural tube defects, including a reduction in head size, cyclopia, reduction of the neural crest territory at neurula stages, and craniofacial malformations. They suggested excess retinoic acid as the mechanism of toxicity. However, we suspect that both impaired DNA repair mechanisms and impaired folate one-carbon metabolism (FOCM) may also play a rôle.

Polynucleotide kinase 3-phosphatase (PNKP) plays an important rôle in DNA repair. As its name implies, it is both a phosphatase and a kinase, and therefore can be expected to be disrupted by glyphosate in both of its enzymatic rôles. Mutations in PNKP have been shown to cause microcephaly, seizures and defects in DNA repair [300, 301].

Disrupted FOCM is an established risk factor for impaired neural tube closure leading to spina bifida and anencephaly [302, 241, 242]. Low maternal folate during the first trimester has been linked to increased risk to spina bifida, and this has inspired several governments to implement a folic acid enrichment programme for staple foods such as wheat-based products, although it is unclear whether the benefits of such programmes outweigh the risks [303]. Folate is synthesized from chorismate in both plants and gut microbes; chorismate is a product of the shikimate pathway that glyphosate disrupts [304].

FOCM operates in both the cytosol and the mitochondria. In the mitochondria, the reaction produces formate, a precursor to both purine synthesis and methyltetrahydrofolate, which plays an essential rôle in methylation pathways [302]. Impaired methylation capacity in the brain has been linked to autism [305, 306].

We mentioned the glycine cleavage system in the section on glycine riboswitches, where we suggested that impaired methylation capacity and glycine toxicity could arise due to glyphosate's disruption of this system in the gut microbes. An important regulatory enzyme in the GCS is glycine decarboxylase (GLDC). The lysine residue in human GLDC that binds to pyridoxal phosphate is very near a glycine-rich region that is essential for enzyme activity [307]. Embedded in a peptide sequence that is rich in β-turns and random coils, the glycine-rich region maintains shape and flexibility of the active site.

A study on mice with a deficiency in GLDC demonstrated two distinct outcomes: neural tube defects; and hydrocephalus with enlarged ventricles and non-ketotic hyperglycinaemia [243]. Autism, attention-deficit hyperactivity disorder (ADHD) and schizophrenia have all been linked to enlargement of the ventricles in the brain [308]. Children with prenatal mild ventriculomegaly had significantly larger cortical grey matter than controls and a large ratio of grey matter to white matter, both of which are features of autism [309]. Whole-genome sequencing applied to ASD families revealed links between autism and defective versions of the aminomethyl transferase gene (AMT) [310], another gene involved in glycine cleavage and linked to nonketotic hyperglycinaemia [311]. A case study concerned a boy with transient neonatal nonketotic hyperglycinaemia and autism [312]. Thus, it appears that autism, hyperglycinaemia and neural tube defects are all tied to impaired glycine cleavage and methylfolate deficiency, which can be explained by glyphosate's antibiotic effects as well as its interference with glycine riboswitches and with GLDC enzymatic action.

Another decarboxylase, besides GLDC, with a conserved active-site lysine near a glycine-rich sequence is ornithine decarboxylase (ODC) [313]. This enzyme is essential for the synthesis of spermidine and spermine, which stabilize DNA structure and assist in DNA repair mechanisms. Lack of ODC leads to apoptosis in embryonic mice following DNA damage [314]. Seizures, which are associated with autism [315], lead to an increased synthesis of ODC [316]. Could this be a compensatory reaction to diminishing activity in the context of glyphosate substitution for glycine in the active site?

Vanishing white matter (VWM) disease is a rare leukoencephalopathy caused by mutations in genes encoding the five subunits of eukaryotic translation initiation factor eIF2B [317]. In advanced cases, the white matter in the brain almost completely disappears, presenting a signal indicative of cerebrospinal fluid. Symptoms can include microcephaly, impaired swallowing, failure to thrive, epilepsy, growth retardation, dysgenesis of the ovaries, pancreatic abnormalities, hypoplastic kidneys, hepatosplenomegaly and cataracts, in addition to the leukoencephalopathy [318]. Increased levels of cerebrospinal glycine are a marker for the disease [318, 319], which may indicate neuroexcitotoxicity. A study of the genetic markers for several individual cases revealed mutations localized to two distinct regions containing highly conserved glycines [318]. One contained a single conserved glycine and the other exhibited the pattern GXXGXG.

The glycine receptor class (GlyRs) is a member of a family of ligand-gated ion channels. Glycine receptor activation is required for receptor clustering in spinal neurons, and is important in synaptogenesis [320]. This receptor is widely distributed in the nervous system, particularly in the spinal cord and brainstem [321]. Glycinergic inhibition plays an important rôle in motor

control, pain sensitization and respiratory rhythm [322]. It has been proposed that glyphosate may interfere with GlyR through glycine mimicry, and that this may increase risk to autism [13].

However, glyphosate may also operate at the level of residue substitution for glycine in the peptide sequence. An *in vitro* study on the human isoform by Vandenberg et al. has confirmed that there is a conserved glycine residue, gly160, that forms part of the binding site and helps maintain the tertiary structure for binding [323]. Several mutations in GlyR α1 G160 significantly decrease the potency of glycine as an inhibitor, likely through disruption of glycine binding within the ligand-binding pocket [322].

9. IMPAIRED DEVELOPMENT AND INFERTILITY

A recent study by Coullery et al. has shown that glypyhosate causes irreversible abnormal growth and delayed development in neuronal cells taken from embryonic rats [324]. Cells exposed to sublethal levels of glyphosate exhibited shorter and unbranched axons and less complex dendritic arbours compared to controls. A deeper look into the underlying mechanism of toxicity revealed a decrease in WNT5a signaling, as well as downregulated Ca^{+2}/calmodulin-dependent protein kinase II (CaMKII) activity.

A possible mechanism by which CaMKII might be inhibited by glyphosate is through substitution of glyphosate for one of the highly conserved glycines near ser26. Ser26 is situated within a conserved stretch of nine residues (LGKGAFSVV) that constitute the upper lid of the ATP-binding site in the canonical kinase fold [325]. An intricate control mechanism for preventing excessive activity of this autophosphorylating enzyme involves phosphorylation of ser26, which then interferes with ATP binding and disrupts enzymatic activity in a feedback control mechanism. It was shown that replacement of serine with a negatively charged amino acid had the same effect as phosphorylation, inhibiting enzymatic activity. This suggests that the negative charge, repelling phosphate, is the deactivating agent. Replacement of one of the two nearby glycines with glyphosate would have a similar effect, thus explaining the enzyme inhibition that was observed in the Coullery et al. study.

Basigin, also known as extracellular matrix metalloproteinase inducer (EMMPRIN) and as cluster of differentiation 147 (CD147), is a member of the immunoglobulin superfamily, with a structure resembling the putative primordial form of this family. It plays many rôles in the body, particularly in development. Basigin contains a highly conserved glycine residue, gly181, within its extracellular domain, which is crucial for basigin-mediated signaling and chemotaxis [326]. It also has an important protective rôle in Alzheimer's disease, as it suppresses the production of Aβ [327]. Mutant mice lacking this gene showed impaired short-term memory and latent learning, as well as greater sensitivity to electric foot-shock [328]. Basigin is also critical in fetal development. Embryonic mice lacking basigin develop normally prior to implantation, but most of the embryos die around the time of implantation [329]. The male mice that survived into adulthood produced only a small number of spermatids that made it past the metaphase of the first meiosis. The female mice appeared normal but were probably defective in the step of implantation of the fertilized egg.

10. OTHER ENZYMES WITH CONSERVED GLYCINES

Adenosine 5-phosphosulfate kinase (APS kinase) is an important enzyme that participates in purine, selenoamino acid and sulfur metabolisms. In particular, it is the first and rate-limiting enzyme in methionine synthesis by gut microbes. Methionine is an essential amino acid in humans, and it sits at the crossroads of the methylation and transsulfuration pathways. Thus, we depend in part on our microbiome to synthesize methionine from APS. Glyphosate has been shown to deplete methionine levels in plants, which may be due to its ability to substitute for one or more conserved glycines in its polypeptide chain. APS kinase has been shown to be downregulated by a factor of –2.55 in *E. coli* upon exposure to glyphosate [330]. APS synthase contains an absolutely conserved N-terminal glycine [331].

The human equivalent of this enzyme, 3'-phospho-adenosine 5'-phosphosulfate (PAPS) synthase, is bifunctional—it has both a C-terminal ATP sulfurylase domain and an N-terminal APS kinase domain, connected by a short irregular linker [332]. The N-terminal glycine (gly59) is the initiator of a P-loop sequence, which plays an essential rôle in providing conformational flexibility. When the terminal glycine was experimentally substituted with alanine (a conservative substitution), sulfurylase activity dropped to only 8% of the original level [331]. PAPS formation was also disrupted when either the highly conserved gly59 or the highly conserved gly62 were substituted with alanine. The former alteration prevented the formation of the internal APS molecule, and the latter disrupted the final phosphorylation step. It can be expected that the P-loop's flexibility will also be severely restricted with the addition of a methylphosphonyl group to any of the conserved glycines, as would be the case with a substitution of glyphosate for glycine. PAPS plays an essential rôle in activating the usually highly inert sulfate anion to facilitate sulfoconjugation,

important for detoxifying xenobiotics as well as sulfurylation of sterols, polyphenols and neurotransmitters.

There are almost surely many enzymes with conserved glycines that we have not yet identified, which are also likely to be disrupted by glyphosate substitution for glycine. For example, the 65-amino acid γ subunit of Na,K-ATPase in kidney has a conserved glycine residue at position 4 which, if mutated to arginine or lysine, leads to an impaired ability to oligomerize [333]. This defect causes renal hypomagnesaemia, due to impaired magnesium reuptake in the renal tubules [334]. Acyl phosphatase, an active enzyme in muscles, enhances Na,K-ATPase activity [335], and a defective form could lead to impaired muscle function and heart failure [336]. Acylphosphatase has six conserved glycines [22]. One of them, gly15, is important for enzyme catalysis. The other five are suspected to play a rôle in preventing protein aggregation.

HapR is a quorum-sensing master regulator in *Vibrio cholerae*, controlling a wide range of physiological activities. In particular, it represses biofilm development and the production of primary virulence factors [337]. HapR has a conserved hinge glycine residue (gly39) that regulates its DNA binding ability, which is necessary for its regulatory control. Substitution of asparatate for gly39 renders the molecule nonfunctional.

Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase is the enzyme that is suppressed by statin drugs to reduce serum cholesterol levels. The enzyme contains a glycine-rich region in the C-terminal section of the catalytic domain [338]. Necrotizing autoimmune myopathy (NAM) is a newly recognized condition characterized by idiopathic inflammatory myopathy, associated with necrosis in myocytes despite the absence of notable inflammation. This condition is associated with statin drug therapy, and a notable feature is that termination of statin therapy often does not alleviate symptoms [339]. Increased protein synthesis of HMG Co-A reductase can be expected following its suppressed activity level by statin drugs, and it is also upregulated in regenerating fibres following injury. Thus, it can be argued that overproduction of HMG Co-A reductase provides a greater opportunity for incorporating glyphosate into the enzyme, displacing conserved glycines. This would result in a malfunctioning of the enzyme and, possibly, also an autoimmune reaction to it due to impaired ability to metabolize damaged versions of the protein.

11. GLUFOSINATE: ANOTHER AMINO ACID ANALOGUE

Glufosinate, like glyphosate, is a broad-spectrum herbicide that may derive most of its toxicity from the fact that it is an amino acid analogue of glutamate [340]. In plants it inhibits glutamine synthetase, leading to a complete breakdown of ammonia metabolism.

Glufosinate adversely affects central nervous system development in both mice and rats. Glufosinate exposure to mouse embryos at different stages of development caused great disturbances to the nervous system [341]. Mouse embryos exposed to glufosinate at days 8 and 9 developed hypoplasia in the forebrain and visceral arches. Day 10 embryos exposed to glufosinate exhibited cleft lips as well as hypoplasia, along with significant cell death in the brain vesicle and neural tubes. Glufosinate inhibited the differentiation of midbrain cells in day 12 embryos.

Rats exposed to low doses of glufosinate in the first week of life were tested at six weeks and found to have an enhanced response to kainic acid, which stimulates glutamate receptors in the brain [342]. Glufosinate exposure of mouse dams has been shown to induce autistic-like behaviour in the pups [343]. Glutamate is a major excitatory neurotransmitter, and disrupted glutamate activity in the brain has been linked to autism [344].

Genetic defects for the encoding of the enzyme, asparagine synthetase, have been linked to microcephaly [345]. Asparagine synthetase has a conserved glutamate residue that is essential for its function [346]. There is a conserved glutamate residue in the first transmembrane domain in the entire family of major intrinsic protein (MIP) channels, which includes mammalian aquaporins. An equivalent neurogenic transmembrane protein in *Drosophila* is crucial for neuroblast determination during development [347].

Mutations in a conserved glutamate residue in the sulfonylurea receptor can result in either hyperinsulinism or neonatal diabetes [348]. Symptoms of neonatal diabetes include hyperglycaemia, failure to thrive, dehydration and ketoacidosis, which may lead to coma [349]. An absolutely conserved glutamate (E418) in all voltage-gated potassium channels has been shown to be critical to control the rate of slow inactivation [350].

Glutamate plays an essential rôle in ATP hydrolysis; DNA replication, which depends on ATP, is likely to be impaired if glufosinate can substitute for glutamate in peptides. The Walker B motif is a distinct sequence pattern found in ATP-binding proteins. It includes a conserved glutamate that is essential for ATP hydrolysis [351]. Replication factor C is a clamp loader that assists in the process of second-strand DNA synthesis. It has an absolutely conserved glutamate residue in a Walker B motif that is required for ATP-dependent ligand binding activity [352].

12. SUMMARIZING DISCUSSION

In this paper, we have reviewed the biological function of a large number of proteins containing conserved glycine residues and/or glycine-rich regions, in the light of the concept that glyphosate could be randomly substituting for glycine in these peptides, causing diverse negative consequences. There is strong evidence that glyphosate's mechanism of action includes an ability to substitute for glycine during protein synthesis. In fact, this can explain a large number of known effects of glyphosate on plants, microbes and eukaryotes, which are otherwise difficult to explain. For example, glyphosate's interference with oxidative phosphorylation [82] can now be easily understood through disruption of COX [89]. The disruption of PEPC that leads to impaired nitrogen fixation in plants exposed to glyphosate [226] is also explained through glyphosate substitution of an invariant glycine residue at the C-terminal. Glyphosate has been shown to inhibit iron uptake, and this may be due to both reduced synthesis of siderophores and impaired function of transporters of iron-carrying siderophores [257]. This may also directly explain the renal tubular disease that has become an epidemic among agricultural workers exposed to glyphosate [262], and which was demonstrated in Monsanto's own chronic long-term studies.

It is remarkable that conserved glycines are found in several of the misfolded proteins that are considered causal in prion diseases, Alzheimer's and Parkinson's diseases, and ALS [183, 184, 194, 209, 221, 222]. Substitution of glyphosate for invariant or highly conserved glycine residues in prion proteins, Aβ, α-synuclein and TDP-43 can explain the formation of the soluble, poorly hydrolysable forms of these pathogenic agents that are considered to be the most toxic species.

Prior research strongly supports the position that glyphosate would cause excessive phosphorylation cascade activity combined with impaired dephosphorylation capacity. This can be expected to lead to many diseased states, including cancer, diabetes and pathogen infectivity, particularly HIV [248], but perhaps most significantly, lung diseases such as pulmonary oedema, asthma and COPD [143].

There are many ways in which glyphosate substitution for conserved glycines could affect metabolism. One is through disruption of insulin signaling, particularly in the glucagon-producing cells in the liver, contributing to the recent worldwide epidemic of type 2 diabetes [80]. Another is through the disruption of glycine metabolism, which will result in a build-up of glycine to toxic levels while at the same time depleting the supply of methyl groups for one-carbon metabolism. This can easily link to spina bifida and other neural tube defects [243]. A third is through interference with the function of COX, which would have huge negative consequences for oxidative phosphorylation in the mitochondria, linked to many chronic diseases [87–89]. A fourth is through the impaired ability to export fatty acids from adipocyte stores, a clear path to obesity [48–50]. Impaired arylsulfatase activity is highly disruptive, as many biologically active molecules are sulfated during transit, and desulfation is a necessary step for activation [180]. The ability to produce sulfate anions to populate the extracellular matrix is also impaired, due to the fact that eNOS, a CYP enzyme, has conserved glycines in two regions, and their substitution by glyphosate can be predicted to cause both impaired ability to bind to caveolin in caveolae and impaired dimer formation [23, 167–170]. These two factors provide a plausible explanation for the well known pathology of superoxide production by eNOS in a "decoupled" state, which cannot be directed as intended towards sulfate synthesis [151, 152].

It is remarkable how well the epidemic of beak deformation in chickadees [103, 104] can be explained through the impaired ability of KEAP1 to bind to the cytoskeleton, leading to constitutive Nrf2 activation and overexpression of keratin synthesis. Since sunflower seeds in bird feeders are routinely sprayed with glyphosate just prior to harvest, there is a straightforward explanation for glyphosate contamination in the birds' diet. Overexpression of keratin also explains the inclusion bodies observed in human livers in association with fatty liver disease.

Non-Hodgkin's lymphoma, AIDS and glaucoma are other conditions whose potential link to glyphosate can be explained via displaced glycine residues in the conserved regions of various proteins [292, 290, 278, 223]. Hypothyroidism, pituitary disorder and adrenal insufficiency are also all potential consequences of displaced glycine residues. Collagen, a key protein in bones and joints, as well as the vasculature, is rich in glycines that are essential for the formation of cross-linkages that maintain the strength and elastic properties of the molecule. It is highly significant that mutations in collagen associated with genetic disorders almost always involve glycine residues [281–284]. This also highlights the essential rôle that glycine molecules play in this protein.

13. CONCLUSION

In this paper, we have shown that glyphosate, as an amino acid analogue of glycine, may be erroneously misincorporated into polypeptide chains during protein synthesis. The research literature documents evidence of severe protein impairment through substitution of conserved glycines by other amino acids. It leads to the